FOR THE RECORD

# Contribution of dipole–dipole interactions to the stability of the collagen triple helix

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**Abstract**

Unveiling sequence–stability and structure–stability relationships is a major goal of protein chemistry and structural biology. Despite the enormous efforts devoted, answers to these issues remain elusive. In principle, collagen represents an ideal system for such investigations due to its simplified sequence and regular structure. However, the definition of the molecular basis of collagen triple helix stability has hitherto proved to be a difficult task. Particularly puzzling is the decoding of the mechanism of triple helix stabilization/destabilization induced by imino acids. Although the propensity-based model, which correlates the propensities of the individual imino acids with the structural requirements of the triple helix, is able to explicate most of the experimental data, it is unable to predict the rather high stability of peptides embedding Gly–Hyp–Hyp triplets. Starting from the available X-ray structures of this polypeptide, we carried out an extensive quantum chemistry analysis of the mutual interactions established by hydroxyproline residues located at the X and Y positions of the Gly–X–Y motif. Our data clearly indicate that the opposing rings of these residues establish significant van der Waals and dipole–dipole interactions that play an important role in triple helix stabilization. These findings suggest that triple helix stabilization can be achieved by distinct structural mechanisms. The interplay of these subtle but recurrent effects dictates the overall stability of this widespread structural motif.

**Keywords:** collagen; triple helix; protein–protein association; imino acids; quantum chemistry

**Supplemental material:** see www.proteinscience.org

The definition of the molecular basis of collagen triple helix stability has hitherto proved to be a difficult task, despite the rigid structure and the simplified sequence of this protein. Particularly puzzling is the decoding of the mechanism of triple helix stabilization/destabilization induced by imino acids. While important data on the role played by the sequence in collagen stability are available (Brodsky and Persikov 2005), the effects produced by proline hydroxylation (or fluorination) on collagen triple helix stabilization/destabilization are so far not established (Inouye et al. 1982; Holmgren et al. 1998; Berisio et al. 2002; Jenkins and Raines 2002; Brodsky and Persikov 2005). This is somewhat frustrating taking into account the structural similarity of proline with hydroxyproline (and fluoroproline) and the strong impact that proline hydroxylation has on real collagen stability (Burjanadze 2000). Several studies have indeed demonstrated that the effects of proline hydroxylation on the triple helix are diversified, depending on the position of proline in the Gly–X–Y sequence motif and on the diastereoisomer produced ([4R,2S]-hydroxyproline, Hyp or [4S,2S]-hydroxyproline, hyp). In particular, replacement

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*Abbreviations:* Hyp, (4R,2S)-hydroxyproline; hyp, (4S,2S)-hydroxyproline; Flp, (4R,2S)-fluoroproline; flp, (4S,2S)-fluoroproline; Hyp(X), Pro(X), hydroxyproline, and proline located at the X position; Hyp(Y), Pro(Y), hydroxyproline, and proline located at the Y position.

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of Pro residues located at the X or Y positions of Gly–Pro–Pro triplets with Hyp leads to destabilization or stabilization of the triple helix, respectively (Inouye et al. 1982). On the other hand, the replacement of Pro residues with hyp has destabilizing effects in both the X and Y positions (Inouye et al. 1982). Over the years, a number of models have tried to explain the dependence of triple helix stability on proline hydroxylation (Inouye et al. 1982; Bella et al. 1995; Holmgren et al. 1998, 1999; Vitagliano et al. 2001a,b). Taking into account the different conformations that imino acids generally assume when located at the X (down state) or Y (up state) position and the intrinsic preference of Hyp for the up state (Holmgren et al. 1999; Improta et al. 2001; Vitagliano et al. 2001a, b), a propensity-based model, able to explain both the stabilizing and destabilizing effects of Hyp, has been proposed (Vitagliano et al. 2001a,b).

Although this model is able to provide a satisfactory explanation for polypeptides containing a single proline derivative per triplet, some conflicting data arise from the analysis of systems containing groups that can potentially interact in the triple helix structure. In the framework of the propensity-based model, it is not possible to provide a direct explanation for the stabilizing effects of Hyp when located in the X position in some specific contexts, as has been found in $(Gly–Hyp–Thr)_{10}$ (Bann and Bachinger 2000; Mizuno et al. 2003) and $(Gly–Hyp–Hyp)_{10}$ (Persikov et al. 2003; Berisio et al. 2004; Mizuno et al. 2004; Doi et al. 2005). Neither of these effects can be solely attributed to the higher tendency of Hyp to stabilize the *trans* conformers, since Hyp–Pro–Gly triplets have destabilizing effects on the triple helix. The relatively high stability of the above compounds could be tentatively ascribed to some compensatory favorable interactions between the side chains of residues at the X and Y positions, but the nature of these effects has not yet been established. For $(Gly–Hyp–Hyp)_{10}$, it has been proposed that the two Oδ groups of the facing Hyp residues could form direct hydrogen bonding interactions (Berisio et al. 2004). However, the structural determinations of peptides embedding the Gly–Hyp–Hyp motif have shown that this kind of interaction occurs only occasionally (Kawahara et al. 2005; Schumacher et al. 2005; G. Wu, K. Noguchi, K. Okuyama, K. Mizuno, H.P. Bachinger, unpubl.), (PDB code 2D3H). Indeed, in the vast majority of the cases, the structure of these polypeptides is characterized by an up puckering of Hyp residues located at both the X and Y positions. Attempts to explain this rather unexpected finding have invoked stabilizing interactions mediated by water molecules. In particular, a role for a water bridge linking the Oδ group of the Hyp residue and a carbonyl group of the same chain has been proposed (Schumacher et al. 2005). However, since similar networks could be formed in peptides containing Gly–Hyp–Pro motifs (Inouye et al. 1982; Jiravanichanun et al. 2005), this hypothesis cannot explain the destabilizing effects caused by Hyp in these latter contexts. Therefore, no satisfactory explanation for the rather high stability of peptides embedding Gly–Hyp–Hyp triplets is available. In order to shed some light on the importance of local nonbonding interactions between packed residues in triple helix stabilization, we resorted to quantum mechanical (QM) calculations, by computing the ring–ring interaction energy in different proline-like model systems. These calculations provided clear insights into the structural bases of the extra stability of triple helices containing Hyp–Hyp–Gly triplets.

## Results and Discussion

One of the most intriguing consequences of the up-up puckering of the two Hyp residues found in structures containing Gly–Hyp–Hyp motifs is the tight packing of the HypY side chain against the HypX ring (Kawahara et al. 2005; Schumacher et al. 2005; Fig. 1A). The nonbonded distances between the Oδ atom of HypY (OδY) and the Cγ atom of Hyp (CγX) range from 3.4 to 3.9 Å, with an average value of ~3.5 Å (Supplemental Fig. S1). Notably, similar tight packing interactions are also found in small-molecule structures embedding Hyp residues (Supplemental Fig. S2). To quantify the impact of these interactions on triple helix stability, we carried out QM calculations on eight different proline-like model systems (Fig. 1; Table 1), using a representative Hyp(X)–Hyp(Y) pair, with a (OδY)–(CγX) distance very close to the average, as a starting model. Hence, in all cases the two facing rings were both in an up state. No alternative conformation of the side-chain rings (e.g., down state) was considered since down puckering (due to the interplay between main-chain/side-chain dihedral angles of imino acids) would have been characterized by different backbone dihedrals (Improta et al. 2001) and, therefore, by different interactions (hydrogen bonds and van der Waals) occurring in the triple helical motif. The results of the QM calculations have been complemented with electrostatic calculations.

As shown in Table 1, both MP2 and PBE0 calculations indicate that dimers are significantly more stable than isolated monomers, suggesting that the packing between two pyrrolidine rings receives substantial contribution from nonbonding interactions. Not surprisingly, absolute values of interaction energies are larger in the MP2 calculations, which are more suitable than PBE0 for treating dispersion interactions (see Materials and Methods).

Interestingly, the presence of Oδ groups both in the X and Y positions significantly contributes to the stability of the ring packing. Compared with Pro(X)–Pro(Y), the entity of this stabilization is 0.62 and 0.32 kcal/mol in

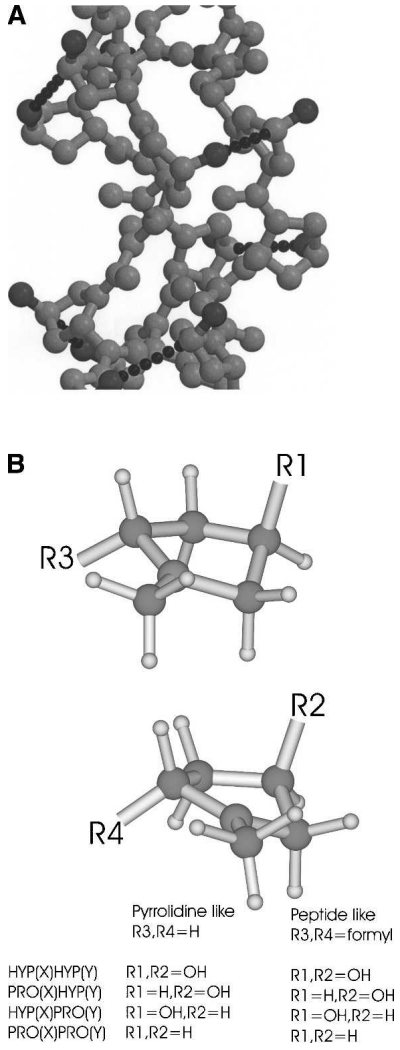



**Figure 1.** (*A*) Snapshot of $(Gly–Hyp–Hyp)_n$ crystal structure (PDB code 1YM8). (Black) Cγ–Oδ Y distances, (dark gray) the Hyp Oγ atom. (*B*) Model compounds used for calculations.

MP2 and PBE0, respectively (Table 1, pyrrolidine-like). This energetic contribution is comparable to that associated with the gauche effect invoked to explain the overstabilization of the up conformers of Hyp residues. Even if Pro(X)–Hyp(Y), whose Oδ group points toward the opposite pyrrolidine ring, could in principle receive additional favorable contributions from hyperconjugative interactions between the oxydryl lone pairs and the CH σ* orbital, its stability is similar to that of Pro(X)–Pro(Y) and Hyp(X)–Pro(Y) and noticeably smaller than that of Hyp(X)–Hyp(Y) (Table 1). Our calculations thus indicate that the extra stabilization of Hyp(X)–Hyp(Y) in Hyp–Hyp–Gly is not due to favorable Oδ(Y)···pyrrolidine(X) nonbonding interactions but to the interaction between the two aligned Cγ–Oδ groups. The dipole–dipole interactions here identified likely dictate the conformation adopted by Hyp in the X position. Indeed, in the absence of Hyp in Y, as in Hyp–Pro–Gly contexts, Hyp(X) adopts the down state required by triple helix restraints (Jiravanichanun et al. 2005). Test calculations starting from dimers characterized by larger (OδY)–(CγX) distances (3.69 Å, residues B16 and G17 in 1YM8) indicate that the above picture does not qualitatively change when the inter-ring distance increases, despite a decrease, as expected, in the energy differences between the peptides. In this case, Hyp(X)–Hyp(Y) is more stable than Pro(X)–Hyp(Y) by 0.1 kcal/mol and than Pro(X)–Pro(Y) by 0.6 kcal/mol (MP2/6 − 311 + G(d,p)//PBE0/6 − 31G(d) calculations).

These results are confirmed by calculations on peptide-like systems, where formyl groups at the N terminus mimic the peptide groups of the polypeptide chains (Fig. 1B). Interestingly, the computed dimerization energies are significantly larger than for pyrrolidine-like rings (see Table 1), indicating that the favorable dipole–dipole interactions between the peptide groups may be another influential factor for explaining the overall triple helix stabilization. Hyp(X)–Hyp(Y) is predicted to be the most stable, confirming that dipole–dipole interactions generated by Cγ–Oδ groups play a significant role in the stabilization of Hyp pyrrolidine groups oriented as in the triple helix. The occurrence of a clear dipole–dipole interaction is also confirmed by the analysis of the Cγ–Oδ bonding distances, which are larger in Hyp(X)–Hyp(Y) (Supplemental Table S1). Indeed, the CO bond distance in the carbonyl group is slightly longer (leading to a larger bond moment) in the presence of a Cγ–Oδ(Y) group, i.e., in Hyp(X)–Hyp(Y) and Pro(X)–Hyp(Y). Analogously, the longest Cγ–Oδ bond distance is found in Hyp(X)–Hyp(Y), confirming that the dipolar interactions between those two groups on up puckerings are stabilizing. Interestingly, in this latter compound the Cγ–Oδ bond distance of Hyp(X) is slightly shorter, in line with the repulsive electrostatic interaction with the carbonyl group of the peptide in Y. This Cγ–Oδ distance remains shorter in Hyp(X)–Pro(Y), where the stabilizing interaction with the Cγ–Oδ in Y is missing.

In peptide-like systems, Pro(X)–Hyp(Y) is noticeably more stable than Hyp(X)–Pro(Y) and Pro(X)–Pro(Y). The favorable interaction energy of the up-up Pro(X)–Hyp(Y) state is in line with previous calculations (Improta et al. 2002) and with the observation that Pro(X) occasionally adopts an up state in collagen-like peptide structures (Okuyama et al. 2007).

The favorable interaction energies of the Hyp–Pro and Hyp–Hyp compared with Pro–Pro and Pro–Hyp clearly indicate that hyperconjugative-type interactions play an important role in these systems. In the case of Hyp–Hyp, dipole–dipole interactions provide some extra stabilization.

The above picture is fully confirmed by M05–2X calculations (see Table 1), which predict the same stability

**Table 1.** *Interaction energy (in kcal/mol) corrected for the basis set superposition error for different dimers (see Fig. 1)*

| | HYP(X)–HYP(Y) | | | PRO(X)–HYP(Y) | | | PRO(X)–PRO(Y) | | | HYP(X)–PRO(Y) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | MP2 | PBE0 | M05–2X | MP2 | PBE0 | M05–2X | MP2 | PBE0 | M05–2X | MP2 | PBE0 | M05–2X |
| Pyrrolidine-like | −2.76 | −0.66 | | −2.26 | −0.25 | | −2.14 | −0.34 | | −2.36 | −0.40 | |
| Peptide-like | −4.41 | −1.74 | −4.75 | −4.12 | −1.59 | −4.46 | −3.41 | −0.89 | −3.43 | −3.48 | −0.86 | −3.50 |

MP2/6–311+G(d,p), PBE0/6–311+G(2d,2p), and M05–2X/6–311+G(2d,2p) calculations on geometries partially optimized at the PBE0/6–31G(d) level. Calculations were performed using the development version of the program Gaussian03 by freezing the inter-ring arrangement to that of residues B7 and G8 from the PDB entry 1YM8 (OδY–CγX distance 3.46 Å). See Materials and Methods and Supplemental Figure S4.

ordering among the different compounds examined. It is noteworthy that the M05–2X results are extremely similar to those provided by MP2 calculations, confirming the reliability of this latter functional for the treatment of nonbonded complexes. Furthermore, the predicted stability trend does not change if the basis set superposition error (BSSE) is not included in the calculations, since BSSE values for the different dimers are rather similar and increase in the order Pro–Pro < Hyp–Pro < Pro–Hyp < Hyp–Hyp. The computed BSSE values are in the range of 0.37 kcal/mol (Pro–Pro) to 0.56 kcal/mol (Hyp–Hyp) at the PBE0/6 – 311 + G(2d,2p) level and 2.76 (Pro–Pro) to 3.53 (Hyp–Hyp) at the MP2/6 – 311 + G(d,p) level.

In order to get a qualitative estimate of the dipolar contributions to the quantum mechanical interaction energies in all model systems, we computed the electrostatic interaction energy between the monomers, by varying the partial atomic charges on the carbonyl and/or Cγ–Oδ groups (Table 2). The interaction energy of a dimer in which all of the partial atomic charges are equal zero is taken as zero.

When only the charges present in one monomer are considered (see the second row of Table 2), the interaction energy is slightly stabilizing. This suggests that electrostatic interactions contribute to the intrinsic stability of Hyp up puckerings. The interaction energy between the carbonyl bond moments is also negative (see the third row of Table 2), indicating that dipole interactions between the peptide groups could play a significant role in the stabilization of the collagen triple helix. In line with our QM results, the interaction energy between two Cγ–Oδ groups (in an up puckering) is stabilizing (see the fourth row of Table 2). On the other hand, a repulsive interaction between a Cγ–Oδ group in the X position and a carbonyl group in the Y position is observed (sixth row in Table 2). This finding is likely related to the observation that Hyp(X) adopts a down puckering in Hyp–Pro–Gly contexts (Jiravanichanun et al. 2005). Finally, an estimate of the main electrostatic contributions in the peptide models of Hyp(Y)–Pro(X) (−3.20 kcal/mol), Pro(Y)–Hyp(X) (−2.70 kcal/mol), and Hyp(Y)–Hyp(X) (−4.67 kcal/mol) is given in the last three rows of Table 2. The predicted stability trend does not qualitatively change when different values of the atomic charges are used, provided that the direction of the bond moment is maintained. Simple electrostatic calculations thus provide the same stability trend obtained at the MP2 and the PBE0 levels (see Table 1), and the energy differences between the different dimers are similar. These results support our hypothesis that dipolar interactions are important for the stabilization of Gly–Hyp(X)–Hyp(Y)-containing triple helices.

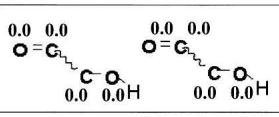
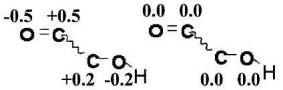
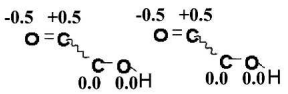
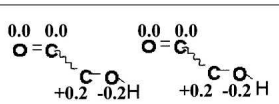
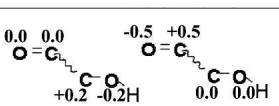
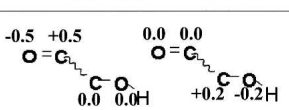
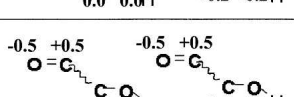
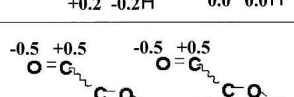
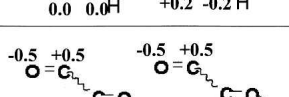
These calculations were also extended to 4R-fluoroproline derivatives. Since no structural information is available for Flp-containing peptides in the triple helix conformation, we assumed for the facing Flp residues the relative experimental orientation of the Hyp residues. As expected, we obtained larger dimerization energies for Flp(X)–Flp(Y) (4.86 kcal/mol) and Pro(X)–Flp(Y) (4.41 kcal/mol) when compared with Hyp(X)–Hyp(Y) and Pro(X)–Hyp(Y). Also, the energy difference between Flp(X)–Flp(Y) and Pro(X)–Flp(Y) is larger than that observed between Hyp(X)–Hyp(Y) and Pro(X)–Hyp(Y). This indicates that dipole–dipole interactions may be stronger in the system containing Flp derivatives. However, it should be pointed out that the predictive value of these data are difficult to assess, since structural differences between Hyp- and Flp-containing triple helical peptides are possible.

## Conclusions

Starting from available X-ray data, we carried out an extensive quantum chemistry analysis of the mutual interactions established by hydroxyproline residues located at the X and Y positions of the Gly–X–Y motif. Our data clearly indicate that the Cγ–Oδ groups of opposing rings establish significant dipole–dipole interactions that play an important role in triple helix stabilization.

In addition, simple electrostatic calculations suggest that the bond moment of Cγ–Oδ of Hyp residues located either in X or Y also interacts with the carbonyl group of the facing residue (Table 2). In particular, the CγY–OδY bond moment forms a stabilizing interaction with the carbonyl group of the residue in X, whereas a slight destabilizing effect is observed for the interaction of CγX–OδX with the carbonyl group of the residue in Y.

**Table 2.** *Estimate of the electrostatic interaction energies in a Hyp(X)-Hyp(Y) dimer obtained by varying the partial charges assigned to the carbonyl and the Cγ–Oδ groups (see computational details)*

| Y X | Energy (Kcal/mol) |
|---|---|
|  | 0.00 |
|  | -1.40 |
|  | -1.61 |
|  | -0.40 |
|  | -0.29 |
|  | 0.27 |
|  | -3.20 |
|  | -2.70 |
|  | -4.67 |

The AMBER energy of a dimer in which all the partial atomic charges are zero (40.5 kcal/mol) is taken as reference energy.

On this basis, our calculations thus indicate that the addition of a hydroxyl group to Pro in the up state has either stabilizing or destabilizing effects if located in Y or in X, respectively. The present data represent the first quantitative evidence that the dipole–dipole interaction generated by the hydroxyl groups may be a factor that contributes to the experimentally observed preference of Hyp for the Y position, a concept that was initially proposed by Holmgren et al. (1999).

It is worth noting that, although the entity of the energetic contributions identified in this work is rather small, the repetitive nature of collagen models (Supplemental Fig. S3) makes the impact of these energies on the triple helix significant.

These findings suggest that triple helix stabilization can be achieved by distinct structural mechanisms. The interplay of these subtle but recurrent effects dictates the overall stability of this widespread structural motif. In line with recent data on collagen heterotrimers (Hodges and Raines 2005; Gauba and Hartgerink 2007), our data suggest that interchain interactions play a role in triple helix stability. Interchain interactions may be responsible for the differences in the propensity scales for the triple helix (Persikov et al. 2000) and polyproline II helices (Rucker et al. 2003; Berisio et al. 2006).

Finally, the present analysis highlights the importance of accurate estimates of the different factors involved in triple helix stabilization. Along this line, dipole–dipole interactions may be exploited for the design of new triple-helix-based biomaterials. It is tempting to believe that the interactions described in this investigation, involving imino acid rings and C–OH-C–OH bond moments, may also play a role in the stabilization of local motifs in globular proteins.

## Materials and Methods

Ground-state partial geometry optimizations are performed in vacuum at the DFT/6–31G(d) level with the PBE0 hybrid functional (Adamo and Barone 1999; Ernzerhof and Scuseria 1999), using PBE0/6–31G(d) calculations. In the geometry optimizations of dimers, intramolecular degrees of freedom were fully optimized whereas the mutual orientation of the constituting monomers and their ring–ring distance were kept frozen to those found in the B7–G8 peptides of the entry 1YM8 of the Protein Data Bank (PDB) (Berman et al. 2000; Schumacher et al. 2005). We choose the B7–G8 peptides as a reference model because they exhibit a CγX–OδY distance (3.46 Å) very close to the average one found in 1YM8 and 1WZB peptides (Supplemental Fig. S1). Test calculations have also been performed starting from the B7–G17 peptides, which exhibit a larger CγX–OδY distance (3.69 Å).

Energies were refined by single-point calculations at the PBE0/6 – 311 + G(2d,2p) level. Despite the absence of adjustable parameters, PBE0 provides accurate results for a number of physico-chemical observables in several systems, including polypeptides (Langella et al. 2002; Improta et al. 2005; Barone et al. 2006). In particular, PBE0 has already been successfully applied to the study of collagen-like polypeptides containing proline derivatives (Improta et al. 2001, 2002). Furthermore, it can provide a quite satisfactory description of the subtle balance of nonstandard hydrogen bonds and weak dispersive interactions contributing to the stabilization of pyrrolidine dimers (Improta and Barone 2004). However, since it is well known that these latter kinds of interactions are usually more reliably treated by the MP2 method, we checked all of our results by single-point MP2/6 – 311 + G(d,p) calculations (Wesolowski et al. 1997; Tsuzuki and Lüthi 2001; Kamiya et al. 2002). We also performed single-point calculations by using the recently developed M05–2X functional, which is based on simultaneously optimized exchange and correlation functionals, including kinetic energy density. This method has shown very good performance in the treatment of dispersion interactions in noncovalent complexes (Zhao et al. 2006). The dimerization energies have been computed as a

difference between the energy of the dimer and the sum of the monomers (in their ground state minima) energies at infinite separation, as schematically depicted in Supplemental Figure S4. The effect of the Basis Set Superposition Error has been taken into account by using the counterpoise method (Boys and Bernardi 1970). All of the calculations have been performed using the Gaussian03 package (Frisch et al. 2003).

The optimized geometry of the Hyp(X)–Hyp(Y) dimer has been used for the electrostatic calculations, and the interaction energy of the dimer has been computed by using the Amber force field, assigning to all the atoms a partial charge of zero, with the exception of the carbonyl and Cγ–Oδ group, whose atomic charges (Table 2) have been estimated by using both the ESP procedure (Besler et al. 1990) and the Natural Bond Orbital (NBO) population analyses (Foster and Weinhold 1980), performed on insulated Hyp molecules at the PBE0/6–31G(d) level. When the partial charge of the oxydryl hydrogen atom is included in the oxygen atom, the charges obtained from the ESP procedure are −0.42 and 0.42 for the carbonyl oxygen and carbonyl carbon, and −0.20 and 0.27 for the Oδ and Cγ atoms of the Hyp side chain, respectively. Similar values are provided by the NBO analysis (C = 0.40, O = −0.53, Cγ = 0.30, and Oδ = −0.28)

## Electronic supplemental material

Table S1 shows bond distances in the optimized models. Figure S1 shows the distribution of CγX–OδY distances derived from the structures 1YM8 and 1WZB, Figure S2 shows a representative example of Hyp–Hyp interactions in the Cambridge Structure Database, Figure S3 shows the crystal structure of $(Gly–4RHyp–Pro)_{10}$, and Figure S4 shows a scheme of the procedure used for the evaluation of the interaction energy of HypX–HypY.

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